POSTHARVEST DECAY OF THE CULTIVATED MUSHROOM
PLEUROTUS ERYNGII CAUSED BY LACTOCOCCUS LACTIS subsp. LACTIS

Y. Zhao, P. Li, H. Hu, Y. Wang, Y. Sun and K. Huang

Institute of Agro-Product processing, Jiangsu Academy of Agricultural Sciences, Nanjing 210014, China

SUMMARY

Pleurotus eryngii is one of the most commercially-grown mushrooms in the world. In this study, the pathogen causing postharvest water-soaked and sunken lesions on the stipes and decay of P. eryngii in Jiangsu (China) was isolated and identified as Lactococcus lactis subsp. lactis based on phenotypic characteristics and the nucleotide sequences of 16S rRNA and gyrB gene. Disease incidence rates were 45.0±8.0% after mushroom storing for 5 days at 20°C in summer, 35.5±6.3% in autumn, 20.5±3.5% in spring, and only 7.5±2.5% in winter. Disease development was closely related to the ambient temperature at which the harvested mushrooms were kept. Thus, cold storage was an effectively method to control the disorder, for the pathogen’s growth was strongly inhibited when mushrooms were grown at a temperature below 10°C and postharvest storage was at 5°C. L. lactis subsp. lactis greatly inhibited the mycelial growth of P. eryngii, and it had obvious extracellular protease activity. To our knowledge, this is the first report that L. lactis subsp. lactis is the causal agent of postharvest decay of P. eryngii.

Key words: postharvest disease, mushroom disease, bacterial pathogen, phylogenetic analysis, antagonism.

INTRODUCTION

Lactococcus lactis, one of the most important members of lactic acid bacteria, is a type of microaerophilic and Gram-positive microorganism with low genomic G+C content (van Hylckama Vlieg et al., 2006; Chen et al., 2011) that occurs in many habitats, such as plant surfaces, milk products, and animal-mucosal surfaces (Makarova et al., 2006; Price et al., 2011). L. lactis is subdivided into L. lactis subsp. lactis, L. lactis subsp. cremoris, and L. lactis subsp. lactis biovar diacetylactis (Stiles and Holzapfel, 1997; Samarzija et al., 2001), whose isolates from various habitats show genetic diversity and occupy different ecological niches (Makarova et al., 2006; Itoi et al., 2009; Price et al., 2011).

L. lactis is long known as a starter of fermented dairy products (van Hylckama Vlieg et al., 2006; Price et al., 2011), thus is usually retained as a beneficial microorganism. However, some of its strains cause neonatal diseases of calves and of aquatic animals (Wang et al., 2008; Chen et al., 2011), and some inhibit the growth of moulds and yeasts (Roy et al., 1996; Stoyanova et al., 2010).

Current evidence shows that the antifungal activity of L. lactis is exerted by its secondary metabolites, such as organic acids, proteinaceous compounds, and alkyl aromatic ketones (Roy et al., 1996; Dalié et al., 2010; Stoyanova et al., 2010). Lactic acid is the major metabolic end product of L. lactis, which acts on the plasma membrane of fungi by eliminating transmembrane proton motive potential, thus increasing its permeability, which would eventually lead to cell lysis (Dalié et al., 2010). However, the mechanisms of action of other antifungal metabolites remain unknown.

Pleurotus eryngii is an edible basidiomycete, and one of the most extensively grown mushrooms in the Mediterranean, Central Europe, Asia and North America (Kim et al., 2008). Recently, a disease was observed on the stipes of postharvest P. eryngii in Jiangsu (China), characterized by a few initial water-soaked and sunken lesions, which expanded on the stipe surface, turning slightly brown. Since to our knowledge, such symptoms had never been reported on postharvest P. eryngii, a study was conducted for isolating and identifying the putative pathogen agent of the disease and investigating its pathogenicity.

MATERIALS AND METHODS

Isolation and culture of bacteria. Lesions excised from symptomatic P. eryngii were homogenized in a sterile mortar with 2 ml of sterile water. Diluted homogenates were plated on 2YT (17 g/l tryptone, 10 g/l yeast extract, and 5 g/l NaCl, pH 7.0) agar plates. After 48 h incubation at 25°C, individual colonies were col-
lected from the plates, and pure cultures were obtained by sub-culturing from a single colony on 2YT plates. After identification, the pathogen was grown at 28°C in MRS medium (10 g/l peptone, 10 g/l beef extract, 5 g/l yeast extract, 20 g/l glucose, 2 g/l K₂HPO₄, 2 g/l dibasic ammonium citrate, 2 g/l sodium acetate, 0.58 g/l MgSO₄×7H₂O, 0.25 g/l MnSO₄×4H₂O, and 1 ml/l Tween 80, pH 6.8) or on MRS agar plates.

Pathogenicity assays. Fresh mushrooms harvested from a commercial farm in Jiangsu province (China), were surface-disinfected with 70% alcohol for 1 min, and dried. Two cross cuts (1.2×1.2 cm) were made on the surface of each stipe with a sterile scalpel before inoculation of a bacterial suspension (20 µl/wound) adjusted to an optical density at 600 nm (OD₆₀₀) of ca. 0.8. Inoculated mushrooms were stored in plastic crispers (Lock&Lock, Korea) at 20°C, the normal shelf-life temperature for mushrooms and observed up to 6 days for lesion development. All experiments were performed in triplicate, and repeated three times.

Phenotypic and biochemical assays. The pathogen was grown on MRS agar plates at 25°C, for observation of the colony morphology whereas the cell morphology was viewed with a light microscope (YS100, Nikon, Japan). Gram reaction, motility, catalase reaction and arginine hydrolyzation assay were performed according to Ling and Dong (1999). The final pH of culture supernatant was measured with a S20 pH meter (Mettler Toledo, Switzerland) after growing the pathogen for 5 days in the MRS medium at 25°C (Itoi et al., 2009).

Carbon sources assays were performed at 0.5% or 1.0% final concentration in PY medium (5 g/l peptone, 5 g/l yeast extract, 8 mg/l CaCl₂, 19.2 mg/l MgSO₄×7H₂O, 40 mg/l K₂HPO₄, 40 mg/l KH₂PO₄, 400 mg/l NaHCO₃, and 80 mg/l NaCl, pH 6.8) according to Ling and Dong (1999). Carbon sources selected based on the diagnostic characteristics of the genus Lactococcus (Ling and Dong, 1999), included ribose, galactose, lactose, maltose, melibiose, raffinose, sucrose, and melilizose.

Molecular identification of the pathogen. Genomic DNA was extracted with a DNA extraction kit (Tiangen, China). A partial DNA fragment of the gyrB gene was amplified using the forward primer fd2 (5’TAGAGTTTGATCATGGCTCAG-3’) and the reverse primer rd2 (5’GGTGTTTGTTTACGCTACG-3’) which were designed based on the sequence of the homologous genes of L. lactis subsp. lactis strains KF147 (GenBank accession No. CP001834.1) and CV56 (CP002365.1). RCR products were purified using a DNA cleanup kit (TaKaRa, China), transformed into pMD19-T simple vector (TaKaRa, China), and sequenced (Invitrogen, China).

Multiple sequence alignments of the partial nucleotide sequences of 16S rRNA and gyrB gene were made using ClustalX 1.83 software (Thompson et al., 1997) and phylogenetic trees were constructed by the neighbour-joining method using MEGA 5.05 software (Tamura et al., 2011). Leuconostoc citreum KM20 (NC_010471.1) and Lactobacillus sakei strain 23K (NC_007576.1) were used as outgroups.

Disease development. Mushrooms collected from the same Chinese locality and farm as above were stored in plastic crispers at 5°C (cold storage conditions) and 20°C (shelf life conditions) and observed daily for disease progression. Disease rates were determined on the third and fifth day at 20°C and at the tenth day at 5°C. Each treatment included four lots of 50 mushrooms each. The same experiment was performed four times within one year.

Growth dynamics of the pathogen at various temperatures. The pathogen was cultivated for 36 h at 28°C in MRS medium. Subsequently, 200 µl of the bacterial culture adjusted to an OD₆₀₀ of ca. 0.5 was inoculated into 20 ml of RSM medium in each 100 ml flask, prior to incubation at 5, 10, 15, 20, 25, and 30°C. Bacterial concentrations were measured at OD₆₀₀ after 6, 12, 24, 36, 48, 60, 72, and 96 h of inoculation. Each treatment included three repetitions and the same experiment was repeated three times.

Determination of extracellular protease activity. To measure the extracellular protease activity of the pathogen, 2 µl of bacterial culture adjusted to an OD₆₀₀ of ca. 0.5 was spotted on MRS agar plates containing 1% (wt/vol) skim milk powder (Zhao et al., 2012). After incubation for 24 h at 25°C, the protease activity was assessed based on the diameters of the clear zones that surrounded the colonies. The experiment was repeated three times.

In vitro antagonism between the pathogen and P. eryngii. An in vitro antagonistic experiment was carried out between the pathogen and P. eryngii on MRS agar plates. A mycelium disc of P. eryngii was positioned at the center of each MRS plate, and incubated at 25°C. When the diameter of the colony was 2 cm, the bacteria were streaked at 1.2 cm distance from the mycelium disc. The antagonistic effect was checked after 3 days incubation at 25°C. The same experiment was repeated three times.
Statistical analysis. Results were analyzed as means of three or four independent experiments ± standard deviation (SD). Tukey’s test was used to identify statistically significant differences (P<0.05). All statistical analyses were done using the SPSS 13.0 software (SPP, USA).

RESULTS

Isolation and identification of the pathogen. A dominant type of bacterial colony was isolated from the lesions on the stipes of postharvest *P. eryngii*. On MRS agar plates, colonies were milk white, obviously convex, and circular with smooth margins. The bacterial isolate named as SLPE1-3 could cause water-soaked and sunken lesions on the stipes of *P. eryngii* (Fig. 1). The lesion was confined to the stipes, and did not extend to the pileus. Such distribution of symptoms tallies with that observed on naturally infected carpophores, showing that the pathogenic behaviour of the isolate SLPE1-3 was the same as that of wild-type bacteria causing the postharvest decay of *P. eryngii*.

Phenotypic characteristics of the pathogen. Strain SLPE1-3 was a Gram-positive and catalase-negative coccus, apparently non motile in MRS agar. The pathogen could metabolize ribose, galactose, lactose, maltose, and sucrose but was unable to hydrolyze L-arginine. The final pH of the cultural supernatant was 4.26±0.02. The biochemical characteristics of SLPE1-3 were similar to those of *L. lactis* subsp. *lactis* (*Lbll*). Based on these results, SLPE1-3 was preliminarily identified as *Lactococcus* species.

Phylogenetic analysis based on nucleotide sequences of 16S rRNA and gyrB. The expected sequences of 16S rRNA (1,417 bp) and gyrB gene (1,486 bp) were amplified from SLPE1-3, and deposited at GenBank under accession Nos. JQ734534 (16S rRNA) and JQ810749 (gyrB). In phylogenetic trees constructed with these sequences, strain SLPE1-3 clustered with those of *L. lactis* subsp. *lactis* (*Lbll*) from cheese starters, vaginas of healthy women, and mung bean sprout (Fig. 2). The phylogenetic analysis suggested that the gyrB gene was able to differentiate the strains of *Lbll* from different environments (Fig. 2B), but 16S rRNA could not (Fig. 2A). Based on this, SLPE1-3 was retained as separate strain of *Lbll*.

Disease progression. Typical lesions were frequently observed on the stipes of *P. eryngii* during postharvest, but they rarely emerged during cultivation. In this study, all none of the mushroom samples developed lesions at 5°C. By contrast, at shelf-life conditions (20°C) mushrooms were easily infected, and their disease rates were significantly diverse in different seasons (Fig. 3). On August 1 (summer), disease rates of the shelf-life mushrooms were 20.5±5.8% on the third day and 45.0±8.0% on the fifth day, respectively (Fig. 3). On January 23 (winter), disease rates were only 3.5±1.8% on the third day and 7.5±2.5% on the fifth day, respectively (Fig. 3), whereas in autumn and spring, the disease rates fell in between those recorded in winter and summer (Fig. 3).

Effect of temperatures on pathogen growth. The growth of *Lbll* SLPE1-3 was severely inhibited when the temperature was below 10°C, and it almost came to a full stop at 5°C (Fig. 4). The growth rate, however, rapidly increased with the rise of temperature when the culture temperature was above 10°C (Fig. 4).

Preliminary analysis of the pathogenesis. The cyto-derm of *P. eryngii* is mainly composed of chitin, proteins and beta-glucans. Our results have shown that *Lbll* SLPE1-3 has strong extracellular protease activity (Fig. 5A), but no obvious chitinase and dextranase activity (data not shown). In addition, strain SLPE1-3 was able to severely inhibit the mycelial growth of *P. eryngii* on MRS agar plates (Fig. 5B), but its cell-free culture filtrate could not (Fig. 6).

DISCUSSION

In recent years, the commercial culture of *P. eryngii* is rapidly expanding in China because of its unique...
flavour, nutritive value, and long shelf life, but pathogenic bacteria seriously threaten this industry (Russo et al., 2003; Kim et al., 2007; González et al., 2009). Up to now, most studies focused on bacterial diseases of growing mushrooms (Kim et al., 2007; Lim et al., 2008; González et al., 2009), whereas postharvest diseases of *P. eryngii* were rarely investigated. The postharvest decay of mushrooms was often attributed to physiological factors and unfit environment. To our knowledge, this is the first report that *Lbll* can cause the postharvest decay of *P. eryngii*.

Although *Lbll* is considered as a kind of safe microorganism for humans (van Hylckama Vlieg et al., 2006), some of its isolates are able to infect and heavily damage organisms such as hybrid sturgeons, newborn calves, moulds, and yeasts (Roy et al., 1996; Stoyanova et al., 2010; Chen et al., 2011). The present study has shown that *Lbll* SLPE1-3 is the causal agent of the lesions on the stipes of postharvest *P. eryngii*, but was unable to infect growing mushrooms and the pileus of the postharvest mushrooms. In addition, lactic acid, the major metabolic end product of *L. lactis*, can cause the lysis of the mycelium of some filamentous fungi (Dalíé et al., 2010). Our results have shown that *Lbll* SLPE1-3 has a strong capability of biosynthesizing lactic acid (pH 4.26). These results demonstrated that the natural iso-

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**Fig. 2.** Comparative analysis of two phylogenetic trees. Numbers at the branches denoted the bootstrap percentages for 1000 replicates. JQ734534 and JQ810749 in parentheses indicated the accession numbers deposited in the GenBank database in this study, and the accession numbers for reference sequences were shown in parenthesis. The scales indicated the evolutionary distance of the nucleotide substitutions per site.
lates of L. lactis subsp. lactis from various environments had special ecological inches and different phenotypic characteristics.

The genetic diversity can affect the phenotypic diversity in the subspecies of L. lactis subsp. lactis (Rademaker et al., 2007; Tan-a-ram et al., 2011; Siezen et al., 2011). In this study, gyrB gene could clearly differentiate four Lbll isolates from different environments (Fig. 2B), but their 16S rRNA sequences shared 100% homology (Fig. 2A), suggesting that the gyrB gene is a more efficient marker for the discrimination of the subspecies within Lbll compared to the 16S rRNA gene.

It is essential to know the growth dynamics of the pathogen under different temperatures before an effective method of controlling postharvest diseases is established. We have shown that the growth of Lbll SLPE1-3 was totally inhibited at 5°C, and severely restricted at 10°C (Fig. 4), in agreement with previous reports.

Fig. 3. Disease investigation of shelf-life P. eryngii. The postharvest mushrooms were stored in plastic crispers at 20°C. Disease rates were investigated on the third day and fifth day, respectively. Values in each band with different markers (*) mean significant difference at P<0.05 level by Tukey’s test. Vertical bars represent standard errors of the means.

Fig. 4. Growth dynamics of the pathogen strain SLPE1-3 under different temperatures. The bacteria were statically cultivated in MRS medium. Culture temperature: (○) 5, (◇) 10, (△) 15, (●) 20, (■) 25, (▲) 30°C. Vertical bars represent standard errors of the means.

Fig. 5. Preliminary analysis of the pathogenesis. A, Determination of the extracellular protease activity of the pathogen strain SLPE1-3 on MRS agar plates containing 1% (wt/vol) skim milk powder. B, In vitro antagonistic experiment between the pathogen strain SLPE1-3 and P. eryngii on MRS agar plates.

Fig. 6. The secondary metabolites of the pathogen strain SLPE1-3 had no obvious inhibition against the mycelial growth of P. eryngii. CK: sterile water; 1: the tenfold cell-free culture filtrate of the strain SLPE1-3; 2: the tenfold cell-free culture filtrate heated for 30 min at 80°C. L. lactis subsp. lactis strain SLPE1-3 was statically cultivated in MRS medium for 48 h at 28°C. The cell-free supernatant was concentrated tenfold as the following method. The cell culture was centrifuged at 15,000 g for 30 min. The supernatant was filtered by a 0.22 µm vacuum filter, and then concentrated by lyophilization. Each hole was filled with 50 µl of the sample, and incubated for 3 days at 25°C. Each treatment included four repetitions. The same experiment was repeated three times.
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(Ahmed et al., 2006; Itoi et al., 2009). In addition, we demonstrated that the growth rate of Lbll SLPE1-3 had a sharply jump above 10°C (Fig. 4) and that the disease rate of postharvest P. eryngii was 7.5-45.0% at 20°C, but cold storage was able to completely control this decay. Currently, cold storage is the first choice for keeping fresh and disease-free many mushrooms in posthar-


